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Characterization of Philippine Drug-susceptible and Multi-drug Resistant *Mycobacterium tuberculosis* Isolates through Combined 15-locus MIRU-VNTR Genotyping and Mutation Analysis of Drug Resistance Genes

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Abstract **- Molecular genotyping, an important strategy to characterize bacterial strains infecting patients, allows identification of *M. tuberculosis* (MTb) complex members with varying responses to anti-mycobacterial therapy. *M. tuberculosis* Interspersed Repeating Units – Variable Number of Tandem Repeats (MIRU-VNTR) is a fast, reproducible and cost-effective PCR-based method capable of differentiating MTb strains. This study focused on evaluating the utility of MIRU-VNTRs to differentiate 54 MTB isolates from the Lung Center of the Philippines (LCP) through amplification of twelve MIRU-VNTRs and three Exact Tandem Repeats (ETRs). Digital codes were determined per isolate through calculation of VNTR repeats and analyzed using the MIRU-VNTR*plus* program (http://www.miruvntrplus.org/MIRU/index.faces). Values of the Hunter-Gaston discriminatory index (HGDI) suggest that five of fifteen (33.33%) MIRU-VNTRs are highly discriminating (>0.75). All MIRU-VNTRs and ETRs except ETRC had HGDI values indicative of good resolving power (≥0.5). Among the LCP isolates, four Mtb clusters closely related to the East African-Indian strain family were identified on the basis of MIRU-VNTR profiles and mutation data on *rpoB, katG* and *gyrA* genes obtained through gene sequencing, which is consistent with previous reports regarding the existence of a distinct Manila family of MTb strains. The said four clusters have been designated as EAI-M1 through EAI-M4, in order of increasing propensity to develop drug resistance. Among these clusters, *rpoB, katG* and *gyrA* mutations were observed that are highly similar to those already reported in literature. Our results demonstrate that a 15-locus MIRU-VNTR genotyping strategy in combination with mutation profiling of drug resistance-related genes could serve as a molecular-epidemiology tool for characterizing and monitoring drug-susceptible and multi-drug resistant MTb strains in the Philippines.**

Keywords: MIRU-VNTR, tuberculosis, TB diagnostics, Hunter-Gaston discriminatory index, genotyping, multi-drug resistance, drug resistance-related mutations

Introduction

Tuberculosis (TB) remains one of the leading causes of morbidity and mortality due to communicable disease. The World Health Organization (WHO) reported approximately 9.0 million new TB cases and 1.1 million deaths due to TB worldwide in 2013, with developing countries such as the Philippines being disproportionately affected [1]. The Philippines is among the 22 high burden countries that account for more than 80% of TB cases globally, with an estimated 75 Filipinos dying of TB every day. Emergence of multi- and extensively drug-resistant TB strains (MDRTB and XDRTB, respectively) further complicates monitoring of TB within the population. In 2013, WHO estimated that 3.7% of new and 20% of previously treated cases were resistant to at least rifampicin (RIF) and isoniazid (INH), which are the two most important first-line anti-TB agents [1], such that second-line anti-TB agents (exemplified by fluoroquinolones) must be added to the anti-TB drug regimen. Because the generation (i.e., doubling) time of MTB in vitro is on the order of 24

hours, reliance on culture-based TB diagnostics often delays the initiation of appropriate treatment regimens among TB patients. Currently, culture-based drug susceptibility testing (DST) is regarded as the gold standard for establishing drug-resistance profiles of MTB [2]. Initial culture and subsequent DST typically enable detection of MDRTB and XDRTB only after 5-6 weeks, during which time disease and development of further drug resistance may progress, possibly with disease transmission as well. Furthermore, contamination of cultures may occur, and MTB may fail to grow in culture even among cases of active TB. Hence, methods for rapid diagnosis of TB are necessary for more timely therapeutic intervention and monitoring the geographic spread of TB.

Rapid, accurate and reproducible yet cost-effective TB diagnosis can be achieved through genotyping with mycobacterial interspersed repeating units-variable number of tandem repeats (MIRU-VNTRs) [13]. This genotyping technique was initially used to identify MTB strains through amplification and analysis of 15 loci in the MTB genome [7] [9] [11]. Recently, a 24-locus platform was introduced to better resolve the relationships among MTB strains.

In the present work, 15 distinct microsatellite regions distributed throughout the MTB genome were used as basis for strain characterization by assignment of 15 digital codes per isolate, corresponding to 12 MIRU-VNTR and three exact tandem repeats (ETRs), in order to analyze the genotypic profiles of contemporary Philippine MTB isolates vis-a-vis mutations in drug resistance-related genes, namely *rpoB* (coding for RNA polymerase subunit B [*RpoB*], which is the target of RIF), *katG* (coding for catalase-peroxidase [*KatG*], with known mutations associated with INH resistance) and *gyrA* (coding for DNA *gyrA*se subunit A [*GyrA*], which is the target of fluoroquinolones).

Methodology

DNA Extraction

MTB isolates used in this study were from the Department of Pathology, Lung Center of the Philippines (LCP). Isolates were grown on Ogawa culture medium in accordance to WHO guidelines. Genomic MTB DNA was extracted using the AllPrep® Qiagen (cat. no. 8004) essentially according to the manufacturer's instructions, with mechanical disruption after chemical lysis. Briefly, loop-full MTB cells were chemically lysed by adding 350 µl RLT buffer (comprising guanidine thiocyanate with freshly added β-mercaptoethanol) and mechanically disrupted by adding sterilized 3-mm glass beads and then vortexing at full speed for two minutes. The resulting mixture was transferred to an AllPrep® DNA spin column fitted with a 2-ml collection tube. Flow-through (recovered and stored at -80^{0}C for subsequent RNA and protein extraction) was eluted using a refrigerated centrifuge pre-cooled at 20^{0}C, at 8,000 rcf for 30 minutes. Genomic DNA was extracted by sequential washing of AllPrep® spin column with buffers AW1 and AW2, followed by centrifugation after each step at 8,000 rcf for 15 seconds and 2 minutes, respectively. Genomic DNA was finally eluted from the spin column by addition of 100 µl buffer EB (preheated to 70^{0}C) followed by centrifugation at 8,000 rcf for 1 minute. Samples were stored at -80^{0}C until use.

PCR Amplification of MIRU-VNTRS

PCR primers were synthesized by Macrogen Inc. (South Korea) having sequences for the 15 MTB genomic loci (12 MIRUs plus ETRs A, B and C) as previously described [2]. For each PCR reaction, a 25-µl reaction mixture was prepared containing 25 pmol of each primer pair, 2.5 µl of PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mix, 0.2 U of TopTaq Qiagen® (cat. no. 200403) and 1 ng of template DNA. The PCR profile used consisted of an initial denaturation step at 95ºC for 12 minutes; 35 cycles each comprising a denaturation step at 94ºC for 30 seconds, an annealing step at 60ºC for 1 minute, and an elongation step at 72ºC for 1 minute; and a final extension step at 72ºC for 5 minutes.

Amplicons were electrophoresed on a 2% agarose gel premixed with 1X Biotium GelRed® Nucleic Acid Gel Stain (cat. no. 41008) at 75 V for 45 minutes using 1X Tris-Acetate EDTA running buffer, along with Vivantis 100-bp ladder molecular weight markers. Gels were analyzed using a BioRad Gel Documentation system to estimate the number of base pairs per amplicon. Calculation of the number of repeats for each MIRU-VNTR locus was performed on the basis of molecular weights of the amplicons.

PCR Amplification of Drug Resistance Genes

PCR amplification of *rpoB*, *katG* and *gyrA* genes was performed using the primer sequences in Table 1. Twenty-five microliter reaction was prepared using Promega GoTaq® Flexi (cat no. M8291) Green Master Mix with five microliters of 2X GoTaq® Green Buffer, 0.5 µl of 10 µM upstream and downstream primers, DNTP and Taq polymerase, 1.5 µl of 25 mM $MgCl_2$, 9 µl of nuclease free water and 7.5 µl of DNA template. The PCR profile used consisted of an initial denaturation step at 95ºC for 12 minutes; 35 cycles each comprising a denaturation step at 94ºC for 30 seconds, an annealing step at 60ºC for 1 minute, and an elongation step at 72ºC for 1 minute; and a final extension step at 72ºC for 5 minutes. PCR products were electrophoresed and base pairs of each amplicon was analyzed prior to DNA sequencing to Macrogen Inc. for DNA sequencing. DNA sequences were analyzed using Molecular Evolutionary Genetics Analysis version 6 (MEGA 6.0) software [23].

TABLE 1: Primer sequences for amplification of drug resistance-related genes

Primer Name	Primer Sequence (5'→ 3')	
	Forward	Reverse
rpoB	GTTCGGGTTCATCGAAACGC	GTCGGAG ATGTTCGGGATGT
katG	GCTTGTCGCTACCACGGA	CCATGAACGACGTCGAAACAG
gyrA	GGATGTTCGGTTCCTGGATGT	CGGCCAGTTTTGTAGGCATC

Statistical Analysis for Allelic Diversity and Discrimination

The Hunter-Gaston discrimination index (HGDI) equation was used to calculate the allelic diversity for each MIRU-VNTR locus, as:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j$$

where D is the index of discriminatory power, a_j is the number of strains in the population which are indistinguishable from the j^{th} strain, and N is the number of strains in the population [6].

Results

DNA sequencing and subsequent analysis of mutations in drug resistance-related genes (Table 2) revealed that 21 of the 54 LCP isolates (38%) harbored missense mutations in the *rpoB* gene. These mutations affect the three codons (corresponding to residues 256, 516 and 531 of *RpoB*) most commonly reported in the literature as conferring RIF resistance.

TABLE 2: Summary of drug resistance-related gene mutations of 54 MTB isolates

GENE NAME	Consequent Protein-level Mutation			Isolate Code(s)
	Nucleotide in MTB H37Rv	Nucleotide Mutation	Consequent Amino Acid Mutation	
rpoB	GAC	GTC	D516V	22
	CAC	TAC	H526Y	09, 50
		CGC	H526R	40
	TCG	TTG	S531L	10, 11, 17, 19, 26, 27, 29, 34, 36, 38, 39, 41, 42, 43, 48, 49, 51, 52, 53, 54
katG	AGC	ACC	S315T	09, 11, 17, 19, 20, 22, 25, 29, 31, 33, 35, 38, 39, 40, 41, 42, 43, 50, 51, 52, 53, 54
		ACA	S315T	42
gyrA	GCG	GTG	A90V	19, 22, 29, 37
	GAC	GGC	D94G	11, 17, 54
		CAC	D94H	51, 53
		AAC	D94N	52

Missense mutations affecting residues 531, 256 and 516 of *RpoB* were found in 81%, 14% and 5% of the *rpoB* mutants. Mutations in *katG* known to be associated with INH resistance were also detected in 43% of the isolates. All the *katG* mutants harbored missense mutations affecting residue 315 of *KatG*. Furthermore, 19% of the isolates harbored missense mutations in *gyrA*, affecting residues 94 and 90 of *GyrA* (60% and 40%, respectively). Of the *gyrA* mutants found, half (namely isolates 11 and 51 through 54) were known to be extremely drug resistant (XDRTB) on the basis of DST results confirming resistance to at least one of the second-line anti-TB agents kanamycin (KAN), levofloxacin (LEV), amikacin (AMK) and capreomycin (CAP).

All the mutations presented in Table 2 have been previously reported in literature on TB isolates from countries other than the Philippines. Moreover, these mutations are regarded as high-confidence mutations strongly associated with phenotypic drug resistance according to the TB Database (http://www.tbdb.org/) [3].

The distribution of drug resistance-related gene mutations among the 54 TB isolates studied is summarized as a Venn diagram in Fig 1 (with color coding as also used for isolate codes in Table 2). The first category (designated as mutation-negative and unrepresented in Fig 1) included 24 isolates for which no mutations were found in *rpoB*, *katG* and *gyrA*, which would be consistent with drug-susceptible MTB. The second category included the 13 isolates found to harbor monoresistance for RIF, INH and fluoroquinolones were found in 13 isolates affecting *rpoB* (in seven isolates), *katG* (five isolates) and *gyrA* (one isolate). The third category includes eight isolates found to harbor double-gene mutations affecting both *rpoB* and *katG*, as would be consistent with MDR TB. The fourth category includes 9 isolates (five of which were confirmed to be XDR TB by DST) with triple-gene mutations affecting *rpoB*, *katG* and *gyrA* as expected for XDR TB.

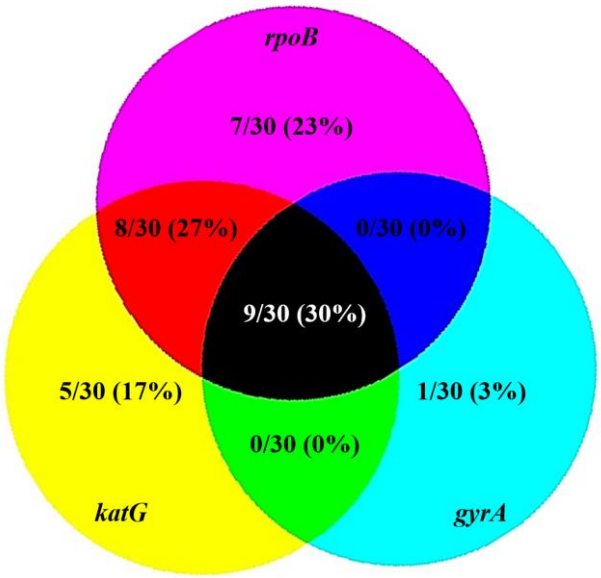

Fig 1: Distribution of mutations in *rpoB*, *katG* and *gyrA* among 30 MTB isolates.

Genotyping of the 54 TB isolates studied using 15-locus MIRU VNTR (Table 3) and analysis of relationships among these strains through generation of a radial neighbor-joining tree using the MIRU-VNTR*plus* program revealed four distinct clusters (Fig 2, with color coding as also used for isolate codes in Table 2 and the Venn diagram in Fig 1). Cluster EIA-M1 isolates lack mutations in all the sequenced drug resistance-related genes and share highly similar MIRU-VNTR profiles with the wild-type H37Rv, which would be consistent with drug-susceptible TB. The remaining three clusters are designated as EAI-M2 through EAI-M4, to reflect the

TABLE 3: Summary of MIRU-VNTR and ETR allele codes of 54 MTB isolates

ISOLATE CODE	M02	M04	M10	M16	M20	M23	M24	M26	M27	M31	M39	M40	ETRA	ETRB	ETRC
01	1	4	6	5	4	6	1	2	2	3	2	2	2	4	0
02	1	4	6	6	4	6	2	2	6	4	3	2	2	4	0
03	1	4	5	5	4	7	1	5	5	3	2	2	1	4	0
04	1	4	5	5	4	7	2	2	5	3	2	1	2	3	0
05	1	1	5	4	4	5	0	2	6	3	2	2	0	0	0
06	1	4	5	4	4	7	1	0	5	3	2	1	2	4	0
07	0	4	5	4	3	6	1	2	5	3	2	1	2	4	0
08	1	4	6	5	4	6	1	2	2	3	2	1	2	4	0
09	1	4	5	4	4	7	2	4	6	4	3	1	2	4	0
10	1	1	4	4	3	6	0	3	6	6	5	2	0	0	0
11	1	1	5	4	4	6	1	3	5	3	2	3	0	0	0
12	1	4	5	4	4	7	2	4	5	4	3	2	2	4	0
13	1	4	4	5	4	7	2	4	4	4	3	1	2	5	0
14	1	4	4	4	4	7	2	4	5	4	3	2	2	4	0
15	0	4	4	4	4	7	2	4	5	4	3	1	1	4	0
16	1	4	4	4	3	8	2	4	5	5	4	1	2	4	0
17	0	1	6	2	3	6	1	3	5	5	4	2	0	0	0
18	0	4	6	3	3	8	3	3	6	4	3	5	1	2	0
19	0	4	6	4	3	8	2	3	6	5	4	4	1	1	0
20	0	1	5	4	3	6	1	2	6	6	5	5	1	0	0
21	0	4	5	4	3	7	2	3	6	4	3	4	1	2	0
22	0	4	6	3	3	7	2	1	6	3	2	4	1	2	0
23	0	4	5	4	3	7	2	3	6	4	3	3	1	1	0
24	0	4	5	3	3	7	2	3	5	5	4	3	1	2	0
25	0	4	6	4	3	7	2	3	6	4	3	3	1	1	0
26	0	4	6	3	3	7	2	2	5	5	4	3	1	2	0
27	0	4	5	4	3	7	2	3	5	5	4	3	1	2	0
28	1	4	5	4	3	7	2	3	5	4	3	3	1	2	0
29	1	4	6	4	3	7	2	4	5	6	5	3	1	2	0
30	1	5	6	3	3	7	2	4	5	4	3	3	1	3	0
31	1	5	6	3	4	7	2	4	5	4	3	3	1	3	0
32	1	4	5	4	5	8	2	4	6	6	5	3	1	3	0
33	0	1	3	4	4	5	2	6	4	5	4	3	0	0	0
34	0	5	3	5	4	6	3	5	4	6	5	4	1	2	0
35	0	5	3	5	3	6	0	5	5	6	2	4	1	1	0
36	0	5	3	4	3	6	2	5	4	5	4	4	1	2	0
37	0	5	3	4	3	5	3	2	2	4	4	4	1	2	0
38	0	1	3	3	3	4	1	5	4	4	3	2	0	0	0
39	0	1	2	3	3	4	1	5	4	3	2	2	0	0	0
40	0	4	3	4	3	6	2	5	3	4	4	3	1	2	0
41	0	1	1	4	3	5	1	3	3	6	5	5	1	0	0
42	0	5	3	4	3	7	3	2	4	3	2	3	1	1	0
43	1	1	2	4	4	5	0	3	5	7	2	5	0	0	0
44	1	5	3	3	3	6	1	4	4	5	4	3	1	2	0
45	1	5	3	4	4	7	2	5	5	4	3	3	1	2	0
46	1	5	3	4	4	8	3	5	6	6	5	3	1	1	0
47	1	5	3	6	4	6	2	6	7	6	5	4	1	2	0
48	1	5	2	4	4	6	3	5	6	4	4	3	1	2	0
49	1	5	6	6	7	7	2	2	3	5	4	2	1	2	0
50	1	5	6	6	7	7	0	2	4	4	3	2	0	2	0
51	1	1	6	5	6	7	0	4	3	4	2	1	0	0	0
52	1	1	7	5	7	7	0	5	3	4	2	2	0	0	0
53	1	1	7	5	6	7	0	5	3	4	2	1	0	0	0
54	1	1	7	4	7	6	0	5	4	4	1	1	0	0	0

progressively increasing proportion of isolates harboring double- and triple-gene mutations per cluster.

Genotyping of the 54 TB isolates studied using 15-locus MIRU VNTR (Table 3) and analysis of relationships among these strains through generation of a radial neighbor-joining tree using the MIRU-VNTR*plus* program revealed four distinct clusters (Fig 2, with color coding as also used for isolate codes in Table 2 and the Venn diagram in Fig 1). Cluster EIA-M1 isolates lack mutations in all the sequenced drug resistance-related genes and share highly similar MIRU-VNTR profiles with the wild-type H37Rv, which would be consistent with drug-susceptible TB. The remaining three clusters are designated as EAI-M2 through EAI-M4, to reflect the progressively increasing proportion of isolates harboring double- and triple-gene mutations (i.e., consistent with MDR and XDR TB, respectively) per cluster. Cluster EAI-M2 consists of 10 isolates, including two isolates with double-gene mutations but no isolates with triple-gene mutations. Cluster EAI-M3 consists of 24 isolates, including two and five isolates with double- and triple-gene mutations, respectively. Cluster EAI-M4 consists of 14 isolates, including four and six isolates with double- and triple-gene mutations, respectively. Hence, the percentage of isolates with either double- or triple-gene mutations in clusters EAI-M1 through EAI-M4 is 0%, 20%, 21% and 77%, respectively. Moreover, neither EAI-M1 nor EAI-M2 contains any isolate with triple-gene mutations, whereas 13% and 43% isolates have triple-gene mutations in EAI-M3 and EAI-M4.

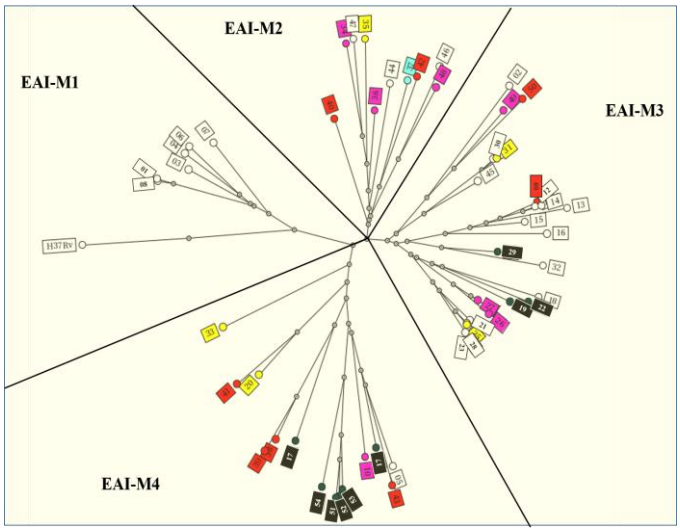

Fig 2: Neighbor-joining tree of wild-type H37Rv strain and 54 MTB isolates from the Lung Center of the Philippines. Isolates lacking mutations in *rpoB*, *katG* and *gyrA* are colored white. Isolates with single-gene mutations in *rpoB*, *katG* or *gyrA* are colored magenta, yellow and cyan, respectively. Isolates harboring double-gene mutations affecting both *rpoB* and *katG* are colored red while isolates harboring triple-gene mutations affecting *rpoB*, *katG* and *gyrA* are in black.

Within the broader phylogenetic context, the relationships among MIRU-VNTR profiles of the 54 MTB isolates together with the other lineages of the MTB complex (data from the MIRU-VNTR*plus* database) is presented in Fig. 3. The isolates are closely related to *Mycobacterium canetti*, which is regarded as an ancestral lineage of mycobacteria [16]. The isolates also clustered near the East African-Indian (EAI) strains, which would be consistent with the emergence of a distinct family of isolates endemic to the Philippines (e.g., separate from the Beijing family of strains).

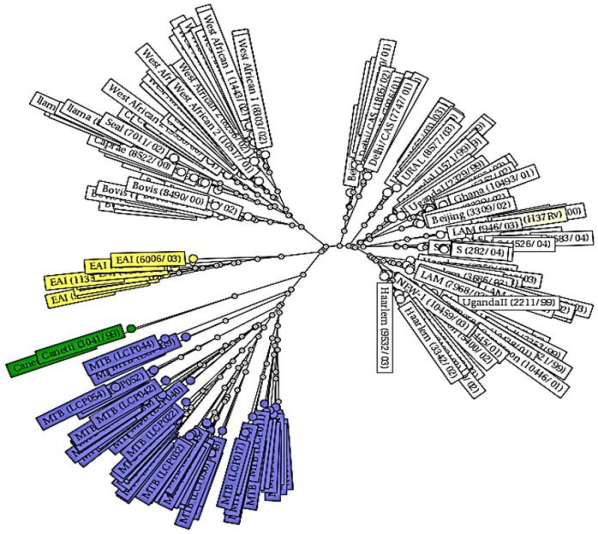

Fig 3. Neighbor-joining tree of 54 MTB isolates (blue) in relation to East African-Indian (EAI) strain family (yellow), *Mycobacterium canetti* (green) and other mycobacterial lineages from MIRU-VNTR*plus* database (white), based on similarity of MIRU-VNTR profiles.

The calculated HGDI of each MIRU-VNTR (Table 4) indicate that five of the 15 gene locus markers (33.33%) have HGDI values >0.75 (i.e., highly discriminating). Overall, the MIRU-VNTRs used in this study have an HGDI >0.5 (0.59) indicative of good resolving power in discriminating among isolates, with the sole exception ETRC (HGDI=0.00).

TABLE 4: Summary of HGDI values of MIRU-VNTRs and ETRs

MIRU	ALLELE NUMBER									HGDI VALUE	DISCRIMINATORY POWER*
	0	1	2	3	4	5	6	7	8		
2	23	31	0	0	0	0	0	0	0	0.50	Moderate
4	0	14	0	0	26	14	0	0	0	0.65	Moderate
10	0	1	3	12	5	15	15	3	0	0.80	High
16	0	0	1	9	30	10	4	0	0	0.64	Moderate
20	0	0	0	26	21	1	2	4	0	0.62	Moderate
23	0	0	0	0	2	5	16	26	5	0.67	Moderate
24	9	12	27	6	0	0	0	0	0	0.67	Moderate
26	1	1	12	13	12	13	2	0	0	0.80	High
27	0	0	3	6	10	20	14	11	0	0.76	High
31	0	0	0	11	23	10	9	1	0	0.73	Moderate
39	0	1	16	16	13	8	0	0	0	0.67	Moderate
40	0	11	13	18	8	4	0	0	0	0.78	High
ETR											
A	13	30	11	0	0	0	0	0	0	0.60	Moderate
B	14	6	2	4	11	1	0	0	0	0.88	High
C	54	0	0	0	0	0	0	0	0	0.00	Low
OVERALL MEAN (MIRU VNTR and ETR)										0.65	Moderate

**HGDI values: <0.5 low, >0.5 but <0.75 moderate, while >0.75 high*

Discussion

Molecular genotyping facilitates identification of mycobacterial strains through comparison of specific genomic regions that serve as genetic markers [10]. This strategy enables profiling of isolates for monitoring the emergence and spread of new strains in a given geographic location for molecular epidemiology [14]. Distinct genotypes of mycobacteria affect various geographic locations [16], respond to treatment regimens differently and vary in their pathogenicity, as exemplified by the MTB Beijing strain [8] [15]. Using a 15-locus MIRU-VNTR genotyping panel including ETRs A, B and C, the present work has established four clusters of MTB isolates from Filipino patients. The MIRU-VNTR profiles thus obtained exhibit a high level of diversity, with the MIRU-VNTR loci apparently polymorphic despite the limited number of alleles that was used with exception of the ETRC.

Standardized 15-locus MIRU-VNTR profiling of MTB isolates has been recommended for improving discriminatory power on the basis of clonal stability and evolutionary rates [5]. In the present work, the high resolving power to discriminate MTB isolates is mainly attributed to MIRU-VNTR loci 10, 26, 27, 40 and ETRB, suggesting that these regions are associated with high evolutionary rates. MIRU-VNTRs with moderate allelic variation account for stability of the genotyping strategy and are equally important for strain identification. High discriminatory indices of MIRU 4, ETR A and B have also been reported by Allix et al., (2006) who performed a 12-locus MIRU-VNTR analysis in genotyping *Mycobacterium bovis* strains. ETRC, with its low HGDI (0.00), probably can be excluded in the further development of genotyping methods for the Philippines setting. The overall HGDI average of 0.65 for the 15-loci MIRU-VNTRs suggests that the composite genotyping method is capable of discriminating Philippine MTB isolates.

Combining MIRU-VNTR genotyping data with *rpoB*, *katG* and *gyrA* mutation profiles revealed that MTB strains endemic to the Philippines appear to comprise a spectrum of clusters (i.e., EAI-M1 through EAI-M4) with respect to propensity for drug resistance. At the extremes of the spectrum are EAI-M1 (lacking any mutations associated with drug resistance, which is typical of drug-susceptible TB) and EAI-M4 (characterized by a preponderance of double- and triple-gene mutations associated with MDR and XDR TB, respectively). EAI-M2 and EAI-M3, which comprise the majority of isolates lacking mutations and of single- and double-gene mutations, may be as intermediate clusters in terms of their divergence from the wild type strain H37Rv. These intermediate clusters demand closer attention insofar as they conceivably could serve as transition clusters comprising isolates having the propensity to develop drug resistance toward MDR and XDR TB. Triple-gene mutants in EAI-M4 confirmed as MDR and XDR TB by DST and/or molecular probe-based assays (data not shown) are the isolates of greatest concern from a global-health perspective.

As an ancient pathogen that afflicting human populations worldwide, MTB has developed a stable association with its host [17]. Various MTB lineages have been identified, namely Indo-Oceanic, East African-Indian, Euro-American, West African 1, West African 2 and East Asian, each endemic to distinct geographic locations [18] [19]. Interestingly, all 54 isolates studied in the present work clustered near but not within the East African-Indian (EAI) lineage, thus supporting findings of Douglas et al., (2003) who identified a unique group of strains endemic to the Philippines (i.e., the Manila family of strains) through restriction fragment length polymorphism (RFLP) [20].

Conclusions

Standard 15-locus MIRU-VNTR genotyping of MTB is useful for discriminating among MTB isolates from patients at the Lung Center of the Philippines, with loci 10, 26, 27, 40 and ETRB found to be highly discriminating. Combination of MIRU-VNTR genotyping data with mutation data on the drug resistance-related genes *rpoB*, *katG* and *gyrA* reveals clustering of the isolates into clusters EAI-M1 through EAI-M4, which form a spectrum of categories with respect to proportion of MDR and XDR TB isolates, such that EAI-M1 consists of isolates lacking mutations in the said genes while EAI-M4 consists mainly of isolates harboring double- and triple-gene mutations associated with MDR and XDR TB, respectively; whereas EAI-M2 and EAI-M3 appear to be transition clusters with potential to develop further drug resistance towards MDR and XDR TB. The isolates all cluster together to form a distinct family of strains most closely related to the East African-Indian lineage, which is consistent with previously published RFLP data suggesting the existence of a distinct Manila family of TB strains. These findings provide basis for further development of tools for molecular epidemiology as applied to the control and elimination of TB.

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